EMBO reports

Knockdown of the mitochondria-localized protein p13 protects against experimental parkinsonism

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 August 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below. Since Esther Schnapp is currently traveling I have temporarily taken over the handling of your manuscript.

As you will see, the referees acknowledge the potential interest of the findings but they also raise a number of - often overlapping - issues that would need to be addressed before publication. Referees 2 and 3 point out that further data on the mitochondrial localization of p13 should be shown and both referees notice a potential interaction with PINK1. Moreover, further data on p13 in the mouse brain/neurons should be shown to strengthen the significance of the findings. Referee 1 suggests to analyse complex I assembly in vitro and in vivo. Moreover, referee 1 and 2 both indicate that the limitations of the potential therapeutic potential should be discussed.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes suitable for publication in EMBO reports. However, given the constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific

Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

In this manuscript, Inoue et al. explored the function of a mitochondria-localized protein p13 and its possible involvement in PD pathogenesis using mitochondrial toxin-induced PD models. In human neuroblastoma cells, p13 knockdown attenuated rotenone induced deficits in mitochondrial membrane potential and complex I activity. Importantly, the authors generated p13 knockout mouse model and showed that p13 heterozygous knockout mice were resistant to MPTP-induced deficits in complex I activity, and prevented MPTP-induced motor deficits and loss of dopaminergic neurons in the substantia nigra. The current finding is interesting and could be potentially important for suggesting a novel therapeutic target for PD. Here I recommend revision of the manuscript with suggested experiments and comments listed below.

1. The authors argue that the protective effects of p13 knockdown is based on the regulation of

complex I, they also mentioned in the text that p13 is possible an assembly factor of complex I, but it remains unclear whether/how complex I assembly is affected by p13 knockdown under toxin-induced conditions. Blue Native-PAGE and Western immunoblot analysis should be performed to check complex I assembly in those conditions in both cellular and mouse models.

2. 80-90% knockdown of p13 in SH-SY5Y cells had no effect on complex I activity and mitochondrial membrane potential under physiological conditions, suggesting that endogenous p13 is rather dispensable for complex I/mitochondrial function. However, p13-/- mice showed high lethality after birth, but it is unclear whether the early lethal phenotypes could be, at least partially, attributed to mitochondrial dysfunction. The authors should check whether complex I assembly /activity and mitochondrial function are affected in p13-/- mice. If so, the data will further support the role of p13 in complex I assembly/activity. If not, the authors should discuss the possible explanations such as non-mitochondrial function of p13 or possible off-target effects from CRISPR knockout mice.

3. Many drug candidates identified from toxin-induced PD models have failed to have reliable disease-modifying effects in subsequent clinical trials, this could be partially attributed to the artifacts generated from toxin-induced systems. The authors should discuss the limitations of the currently used toxin-induced PD models and possible alternatives. To strengthen the current manuscript, the authors can consider to test the effects of p13 knockdown on mitochondrial phenotypes in PD patient fibroblasts. This experiment will help assess the therapeutic potential of targeting p13 in disease-relevant conditions.

4. The authors state that "p13 reduction works in part as an endogenous protective mechanism against PD pathogenesis" based on the observation that p13 expression is downregulated after mitochondria-toxin treatment. But many mitochondria proteins are downregulated under toxin-induced conditions, and the overall change in gene expression of mitochondrial proteins (including p13) suggests it is rather a downstream effect from mitochondrial dysfunction. The authors should adapt the statement and incorporate the other possible explanations.

Referee #2:

This is an interesting report by Inoue and colleagues who demonstrated that the reduction in p13, a novel mitochondrial protein that the investigators have previously identified to be associated with oxidative stress in pancreatic islet cells (Higashi et al., 2015 BBRC), protects against the toxicity induced by the parkinsonian neurotoxins rotenone and MPTP both in vitro and in vivo. Although the mechanism underlying p13-mediated neuroprotection remains unclear, they authors speculated that p13 reduction decreases the sensitivity of complex I to parkinsonian toxicants as the protein normally binds to complex I. Importantly, they showed that p13 heterozygous knockout mice are protected against MPTP-induced neurodegeneration and associated motoric deficits. Overall, these findings are interesting and novel. However, I have several issues regarding the manuscript in its present form, as discussed below:

1. Although the mitochondrial localization of p13 is convincing, exactly where it resides in/on the mitochondria is unclear, i.e. OMM, IMM or matrix? Further, the assumption is that p13 is similarly a resident mitochondrial protein in neurons, which needs to be shown.

2. Fig. 1 & 2: Show an additional cell death marker besides cleaved PARP.

3. Page 8-9: The authors stated that "We demonstrated for the first time that p13 expression was reduced by parkinsonian toxicants both in cell culture and in the midbrain of mice" and showed that the mRNA levels of p13 were indeed reduced in the presence of rotenone and MPP+ treatment (Fig. EV4 A-B & 4A). However, in Fig. 2D and 3D, it is evident that the protein level of p13 is not correspondingly reduced. Instead, p13 protein expression is increased in the presence of rotenone treatment.

4. Fig. 3D: Curiously, in the presence of CCCP treatment, the expression of p13 is dramatically reduced. Interestingly, p13 expression silencing appears to reduce the expression of PINK1 in the presence of CCCP. The authors did not provide possible explanation to these findings. Is there

potential interaction between p13 and PINK1?

5. It is also curious why rotenone despite reducing mitochondria membrane potential (Fig 2C) did not lead to an increase in PINK1 levels.

6. Fig. 3D: Full length PINK1 that accumulates in the presence of CCCP should be around 62-64 kD (not 50kD).

7. Fig. 3C: Show immunoblot for complex I. Same for Fig. 4D.

8. Fig. 3E: Show p13 immunoblot.

9. Where shRNA studies were performed, it is common to have at least two shRNA species to rule out off-target effects. Ideally, a genetic rescue experiment with a shRNA-resistant p13 cDNA should be included.

10. TH-positive neurons should be quantified via stereology, which provides an unbiased measurement and is a standard method for PD animal studies. Typically, Nissl staining is provided alongside. It would also be informative to show the striatal dopaminergic innervation.

11. Fig. 4 & EV3: What is the level of p13 in various regions of the mouse brain following MPTP treatment?

12. The authors suggested that "p13 knockdown could be a candidate drug target for PD" (p. 10). It appears that targeting p13 might be challenging as the authors suggest that this needs to be done before disease progression. Moreover, in their previous report, the authors showed that p13 overexpression is beneficial against type 2 diabetes (Higashi et al., 2015 BBRC). How the authors propose to implement this strategy in the clinical setting for PD is therefore unclear.

Referee #3:

In this manuscript, Inoue et al examine the mitochondrial function of p13 (a protein that they recently identified) in SHSY5Y neuroblastoma cells. Using a p13-deficient mice, they further show that the heterozygous animals are resistant against MPTP-induced motor deficits and dopaminergic neuronal loss.

The manuscript is well-written but a lot of information is missing (from the legends in particular) making it tedious for the reader. The work in mice (in particular the p13 KO mice) is interesting and could be of interest for the EMBO readership. Unfortunately however, most of the experiments have been performed in SHSY5Y neuroblastoma cells.

Specific comments

Figure 1
- In Figure 1A, I assume that the authors have used a FLAG antibody to detect p13 (information missing from the legends)? In Figure 2 and in Figure 4, the authors use an antibody against p13. Can they use it in immunofluorescence to confirm mitochondrial localisation of endogenous p13?
- In Figure 1D, the PARP cleavage blot is not terribly convincing (the western blot is not representative of the histogram). The authors should confirm that apoptosis is increased in their model using other methods.
- In Figure 1D, did the authors use a FLAG or a p13 antibody (again, this isn't clear from the legend)? What is the band in the mock lane? Why is it not present in the p13 o/e lane?
- What is the effect of a rotenone treatment on TMRE, Mito Tracker Green and PARP cleavage in SHSY5Y cells over-expressing p13?

Figure 2
- The authors state, rightly so, that "In the basal condition, the fluorescence intensity of TMRE and the cleavage of PARP were unchanged by the p13 knockdown (Fig 2C and D)". How do they reconcile that with the fact that p13 o/e affects TMRE and PARP cleavage in basal conditions

(Figure 1).
- In Figure 2E, the rescue is very modest. The authors forgot to add the stats between Scr shRNA and p13shRNA + p13 o/e. If there is still a significant difference (which the histogram suggests), claiming that "the restoration of p13 expression significantly reversed the p13 knockdown-induced protective effects on ΔΨm and apoptosis in rotenone-treated cells (Fig 2E and F)" is an overstatement.
- In Figure 2F, again the PARP cleavage is very modest on the Western blot (not representative of the histogram).
Figure 3
- Figure 3B is not very informative and could be moved to supplementary data.
- In figure 3C, all the loading controls are missing. The authors should provide the blot of the input, and the supernatant before/after IP. The authors should try to repeat the experiment with endogenous p13.
- Figure 3D and E: I assume the authors have used whole cell lysates (since they detect actin in all the samples)? I would suggest repeating the experiments with mitochondrial enriched fractions, as it seems that PINK1 accumulation is decreased in CCCP-treated p13 shRNA cells as compared to scr. How long were the cells treated with rotenone and CCCP for? The authors should do short treatment (to detect PINK1 accumulation), and longer treatments, to detect ubiquitination of outer membrane proteins (E.g Mfn) and disappearance of mitochondrial markers. How do the authors explain that p13 signal disappears in scr cells after CCCP treatment?

Figure 4
- The work in the KO mice is interesting. Have the authors assessed TMRM, PARP cleavage, mitophagy etc in mouse neurons from the midbrain, and other brain regions. Experiments from Figures 1, 2 and 3 should be repeated in mice neurons.
- It would be interesting to determine if p13 mRNA levels are decreased in brains from patients with sporadic Parkinson's disease.
- In Figure 4D, again all the loading controls are missing. For example, are the complex I protein levels the same in the +/+ and -/- mice?

1st Revision - authors' response 16 November 2017

Response to Reviewer #1:

In this manuscript, Inoue et al. explored the function of a mitochondria-localized protein p13 and its possible involvement in PD pathogenesis using mitochondrial toxin-induced PD models. In human neuroblastoma cells, p13 knockdown attenuated rotenone induced deficits in mitochondrial membrane potential and complex I activity. Importantly, the authors generated p13 knockout mouse model and showed that p13 heterozygous knockout mice were resistant to MPTP-induced deficits in complex I activity, and prevented MPTP-induced motor deficits and loss of dopaminergic neurons in the substantia nigra. The current finding is interesting and could be potentially important for suggesting a novel therapeutic target for PD. Here I recommend revision of the manuscript with suggested experiments and comments listed below.
[Our Response]
We appreciate the reviewer's positive evaluation of our work and thank her/him for the constructive suggestions. Our point-by-point responses to the comments are as follows.

[Reviewer's comment]
1) The authors argue that the protective effects of p13 knockdown is based on the regulation of complex I, they also mentioned in the text that p13 is possible an assembly factor of complex I, but it remains unclear whether/how complex I assembly is affected by p13 knockdown under toxin-induced conditions. Blue Native-PAGE and Western immunoblot analysis should be performed to check complex I assembly in those conditions in both cellular and mouse models.

[Our Response]
We thank the reviewer for raising a very important issue. To answer the reviewer's question, we performed blue native PAGE and western immunoblot analysis to elucidate whether mitochondrial complex I assembly is affected by p13 knockdown under toxin-induced conditions in cellular and mouse models. We found in both *in vitro* and *in vivo* models that p13 knockdown prevented both

rotenone- and MPTP-induced impairment of mitochondrial complex I assembly, but did not affect the assembly under normal conditions (Fig 3D and 4H in the revised manuscript). We have added the relevant description (page 8, line 28 - page 9, line 1, and page 12, lines 3-5) to the Results and Discussion as follows:

The Results and Discussion (page 8, line 28 - page 9, line 1)
The following sentence was added:
"We then examined the effects of p13 knockdown on complex I assembly in SH-SY5Y cells using blue native PAGE and found that p13 knockdown prevented the rotenone-induced impairment of complex I assembly (Fig 3D)."

The Results and Discussion (page 12, lines 3-5)
"Furthermore, the activity of complex I was maintained in the midbrain of $p13^{+/-}$ mice compared with that in $p13^{+/+}$ mice (Fig 4E)."
was changed to
"The activity and assembly of mitochondrial complex I in the midbrain of $p13^{+/+}$ mice were impaired by MPTP, whereas those processes were unimpaired in $p13^{+/-}$ mice (Fig 4G and H)."

[Reviewer's comment]

2) 80-90% knockdown of p13 in SH-SY5Y cells had no effect on complex I activity and mitochondrial membrane potential under physiological conditions, suggesting that endogenous p13 is rather dispensable for complex I/mitochondrial function. However, p13-/- mice showed high lethality after birth, but it is unclear whether the early lethal phenotypes could be, at least partially, attributed to mitochondrial dysfunction. The authors should check whether complex I assembly /activity and mitochondrial function are affected in p13-/- mice. If so, the data will further support the role of p13 in complex I assembly/activity. If not, the authors should discuss the possible explanations such as non-mitochondrial function of p13 or possible off-target effects from CRISPR knockout mice.

[Our Response]

We appreciate these very helpful suggestions. In response to reviewer's comment, we evaluated the complex I activity in the brain and heart of $p13^{-/-}$, $p13^{+/-}$ and $p13^{+/+}$ mice. In the heart, we found that complex I activity in $p13^{-/-}$ mice was significantly reduced compared with the activity in $p13^{+/+}$ mice. Since cardiomyocytes contain abundant mitochondria and are crucial in cardiac function, the significant decrease in complex I activity in the heart may lead to the early lethal phenotypes in $p13^{-/-}$ mice (Fig EV5I in the revised manuscript). It remains unclear why 80-90% knockdown of p13 in SH-SY5Y cells had no effect on complex I activity under basal conditions. We found that, in contrast to the heart, there were no significant differences in complex I activity in the brain among $p13^{-/-}$, $p13^{+/-}$ and $p13^{+/+}$ mice (Fig EV5H in the revised manuscript). These results suggest that the importance of p13 function may differ between cell types. We have added the relevant description (page 13, lines 18-22) to the Results and Discussion as follows:

The Results and Discussion (page 13, lines 18-22)
The following sentence was added:
"It remains unclear why 80-90% knockdown of p13 in SH-SY5Y cells had no effect on complex I activity under basal conditions. We found that, in contrast to the heart, there were no significant differences in complex I activity in the brain among $p13^{-/-}$, $p13^{+/-}$ and $p13^{+/+}$ mice (Fig EV5H and I). These results suggest that the importance of p13 function may differ between cell types."

[Reviewer's comment]

3) Many drug candidates identified from toxin-induced PD models have failed to have reliable disease-modifying effects in subsequent clinical trials, this could be partially attributed to the artifacts generated from toxin-induced systems. The authors should discuss the limitations of the currently used toxin-induced PD models and possible alternatives. To strengthen the current manuscript, the authors can consider to test the effects of p13 knockdown on mitochondrial phenotypes in PD patient fibroblasts. This experiment will help assess the therapeutic potential of targeting p13 in disease-relevant conditions.

[Our Response]

We agree with the reviewer's comment that there is a gap between the pathologic features in the toxin-induced PD models and those of PD patients; however, we could not obtain PD patient fibroblasts for this study. To circumvent the problem, in addition to the toxin-induced model, we used a non-toxic PD model in which PTEN-induced putative kinase 1 (PINK1), a causal gene for PD, is knocked down (Fig EV1I in the revised manuscript). We found that p13 knockdown significantly prevented the decrease in ΔΨm and the PARP cleavage induced by PINK1 knockdown (Fig EV1J and K in the revised manuscript). These data suggest that p13 knockdown may protect against PD pathogenesis in the non-toxic model as well as in the toxin-induced model; however, we understand the limitations of the currently used PD models. Therefore, we amended the relevant descriptions in the Results and Discussion as follows:

The Results and Discussion (page 7, lines 26-29)
The following sentence was added:
"In addition to the toxin-induced PD model, we also examined the effects of p13 knockdown using a non-toxic PD model in which PINK1 was knocked down (Fig EV1I) and found that p13 knockdown significantly prevented the decrease in ΔΨm and the PARP cleavage in the PINK1 knockdown cells (Fig EV1J and K)."

The Results and Discussion (page 13, lines 7-9)
"Given that PD is a progressive and chronic disease, the therapeutic potential of p13 reduction could also be evaluated by other chronic PD models."
was changed to
"The additional therapeutic potential of p13 reduction, such as lack of tolerance or prevention of the disease progression, would be evaluated via further comparative studies using PD patient samples."

[Reviewer's comment]

4) The authors state that "p13 reduction works in part as an endogenous protective mechanism against PD pathogenesis" based on the observation that p13 expression is downregulated after mitochondria-toxin treatment. But many mitochondria proteins are downregulated under toxin-induced conditions, and the overall change in gene expression of mitochondrial proteins (including p13) suggests it is rather a downstream effect from mitochondrial dysfunction. The authors should adapt the statement and incorporate the other possible explanations.

[Our Response]
We appreciate the reviewer's helpful suggestions. Since, as this reviewer notes, many mitochondrial proteins are downregulated in the PD models, p13 downregulation is suggested to be a downstream effect of mitochondrial dysfunction. Therefore, we have weakened our claims that p13 downregulation protects against parkinsonism. Accordingly, we have revised the title and the descriptions in the Results and Discussion as follows:

The title (page 1, lines 1-2)
"Reduction of mitochondria-localized protein p13 protects against experimental parkinsonism"
was changed to
"Targeted knockdown of the mitochondria-localized protein p13 protects against experimental parkinsonism"

The Abstract (page 3, lines 15-16)
"Taken together, our results suggest that manipulating p13 expression could be a promising avenue for therapeutic intervention in PD."
was changed to
"Taken together, our results suggest that manipulating p13 expression may be a promising avenue for therapeutic intervention in PD."

The Introduction (page 5, lines 6-8)
"Taken together, our results strongly indicate that the reduction of p13 expression acts as a protective factor against PD pathogenesis via the maintenance of mitochondrial function."
was changed to
"Taken together, our results suggest that the reduction of p13 expression acts as a protective factor against PD pathogenesis via the maintenance of mitochondrial function."

The Results and Discussion (page 10, lines 24-28)
"These results indicate that p13 expression in dopaminergic neurons is decreased by parkinsonian toxicants and suggest that p13 reduction works in part as an endogenous protective mechanism against PD pathogenesis."
was changed to
"These results indicate that p13 expression in dopaminergic neurons is decreased by parkinsonian toxicants and suggest that p13 reduction might function as part of an endogenous protective mechanism against PD pathogenesis. Alternatively, many mitochondria proteins are downregulated under toxin-induced conditions [36]; thus, downregulation of p13 may be a downstream effect of mitochondrial dysfunction."

The Results and Discussion (page 12, lines 17-20)
"Thus, these results clearly indicate that the reduction of p13 expression acts as a protective factor against PD-related pathogenesis and suggest that the manipulation of p13 expression could be a novel and beneficial treatment option for PD."
was changed to
"Thus, these results suggest that the reduction of p13 expression can act as a protective factor against PD-related pathogenesis and that the manipulation of p13 expression might be a novel and beneficial treatment option for PD."

Responses to Reviewer #2:

This is an interesting report by Inoue and colleagues who demonstrated that the reduction in p13, a novel mitochondrial protein that the investigators have previously identified to be associated with oxidative stress in pancreatic islet cells (Higashi et al., 2015 BBRC), protects against the toxicity induced by the parkinsonian neurotoxins rotenone and MPTP both in vitro and in vivo. Although the mechanism underlying p13-mediated neuroprotection remains unclear, they authors speculated that p13 reduction decreases the sensitivity of complex I to parkinsonian toxicants as the protein normally binds to complex I. Importantly, they showed that p13 heterozygous knockout mice are protected against MPTP-induced neurodegeneration and associated motoric deficits. Overall, these findings are interesting and novel. However, I have several issues regarding the manuscript in its present form, as discussed below:
[Our Response]
We appreciate the reviewer's positive evaluation of our work and thank her/him for the constructive suggestions. Our point-by-point responses to the comments are as follows:

[Reviewer's comment]
1) Although the mitochondrial localization of p13 is convincing, exactly where it resides in/on the mitochondria is unclear, i.e. OMM, IMM or matrix? Further, the assumption is that p13 is similarly a resident mitochondrial protein in neurons, which needs to be shown.

[Our Response]
We appreciated this valuable suggestion. In response to the reviewer's comment, we performed immunoblot analysis of the subcellular fractions to resolve the cellular localization of endogenous p13. We found that p13 was most abundant in mitochondria-enriched fraction both in SH-SY5Y cells and in the mouse brain (Fig 2A and EV3B in the revised manuscript). Furthermore, to characterize the intramitochondrial localization of endogenous p13, we used digitonin fractionation, in which mitochondria were treated with various concentrations of digitonin for progressive membrane solubilization. We found that p13 showed a similar resistance to digitonin compared with a mitochondrial matrix marker, Hsp60 (Fig 2B in the revised manuscript). Tom20 and Tim23, which are mitochondrial outer and inner membrane markers, respectively, are more sensitive to higher concentrations of digitonin than p13 or Hsp60 is (Fig 2B in the revised manuscript). These results suggest that p13 is mainly localized to the mitochondrial matrix. We added relevant descriptions to the Results and Discussion as follows.
In addition, owing to space limitations, the figures showing decreased *p13* mRNA and protein levels in response to shRNA-mediated p13 knockdown (Fig 2A and B in the original manuscript) were moved to Fig EV1A and B in the revised manuscript.

The Results and Discussion (page 7, lines 2-11)

The following sentences were added:
"To resolve the cellular localization of endogenous p13, we first performed subcellular fractionation experiments and observed that endogenous p13 was most abundant in the mitochondria-enriched fraction (Fig 2A and EV3B). Furthermore, to characterize the intramitochondrial localization of endogenous p13, we used digitonin fractionation, in which mitochondria were treated with various concentrations of digitonin for progressive membrane solubilization. We found that p13 showed a similar resistance to digitonin compared with the mitochondrial matrix marker Hsp60 (Fig 2B). Tom20 and Tim23, which are mitochondrial outer and inner membrane markers, respectively, are more sensitive to higher concentrations of digitonin than p13 or Hsp60 is (Fig 2B). These results suggest that p13 is mainly localized in the mitochondrial matrix."

[Reviewer's comment]
2) Fig. 1 & 2: Show an additional cell death marker besides cleaved PARP.
[Our Response]
We thank the reviewer for raising a very important issue. In response to this comment, we detected apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick-end labelling (TUNEL) method. In accordance with the PARP cleavage, we found that overexpression of p13 increased the abundance of TUNEL-positive cells under basal conditions and exacerbated the rotenone-induced increase in TUNEL-positive cells (Fig 1E in the revised manuscript). Furthermore, p13 knockdown significantly prevented the rotenone-induced increase in TUNEL-positive cells (Fig 2E in the revised manuscript). As expected from the results of a PARP cleavage assay (Fig 2D in the revised manuscript), p13 knockdown did not affect the percentage of TUNEL-positive cells under basal conditions (Fig 2E in the revised manuscript). Accordingly, we have amended the Results and Discussion as follows:

The Results and Discussion (page 6, lines 21-24)
The following sentences were added:
"We also applied the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick-end labelling (TUNEL) method to detect apoptotic cells. We found that overexpression of p13 increased the number of TUNEL-positive cells under basal conditions and exacerbated the rotenone-induced increase in TUNEL-positive cells (Fig 1E)."

The Results and Discussion (page 7, lines 16-20)
"In contrast to control knockdown by scrambled shRNA transfections, p13 knockdown significantly prevented the rotenone-induced decrease in ΔΨm and increase in cleaved PARP (Fig 2C and D). In the basal condition, the fluorescence intensity of TMRE and the cleavage of PARP were unchanged by the p13 knockdown (Fig 2C and D)."
was changed to
"In contrast to control knockdown, p13 knockdown significantly prevented the rotenone-induced decrease in ΔΨm and increases in cleaved PARP and TUNEL-positive cells (Fig 2C–E). In the basal condition, the p13 knockdown did not affect the fluorescence intensity of TMRE, the cleavage of PARP or the percentage of TUNEL-positive cells (Fig 2C–E)."

[Reviewer's comment]
3) Page 8-9: The authors stated that "We demonstrated for the first time that p13 expression was reduced by parkinsonian toxicants both in cell culture and in the midbrain of mice" and showed that the mRNA levels of p13 were indeed reduced in the presence of rotenone and MPP+ treatment (Fig. EV4 A-B & 4A). However, in Fig. 2D and 3D, it is evident that the protein level of p13 is not correspondingly reduced. Instead, p13 protein expression is increased in the presence of rotenone treatment.
[Our Response]
We appreciated this critical comment. In response to this comment, we measured p13 protein levels in both rotenone- and MPP^+- treated cells. We found that 5 mM MPP^+ treatment reduced *p13* mRNA levels by approximately 50% while causing decreased p13 protein levels (Fig EV4B and D in the revised manuscript). In contrast to MPP^+, although 100 nM rotenone treatment reduced *p13* mRNA levels by approximately 20%, p13 protein levels showed a slight, nonsignificant increase under these conditions (Fig EV4A and C in the revised manuscript). The reason for the discrepancy is currently unclear. One possibility is that rotenone may inhibit p13 protein degradation through

impairment of lysosomal functions [35]. Given these results, we have weakened our claims that parkinsonian toxicant-mediated downregulation of p13 protects against parkinsonism (please see the response to Reviewer #1's comment (4)). Accordingly, we have revised the title and the Results and Discussion as follows:

The title (page 1, lines 1-2)
"Reduction of mitochondria-localized protein p13 protects against experimental parkinsonism"
was changed to
"Targeted knockdown of the mitochondria-localized protein p13 protects against experimental parkinsonism"

The subheading in the Results and Discussion (page 10, line 4)
"Neuronal p13 expression is decreased by parkinsonian toxicants"
was changed to
"Neuronal p13 expression in parkinsonian toxin exposure and PD"

The Results and Discussion
The following sentence was omitted:
"These results indicate that p13 expression in dopaminergic neurons is decreased by parkinsonian toxicants and suggest that p13 reduction works in part as an endogenous protective mechanism against PD pathogenesis."

The Results and Discussion (page 10, lines 20-24)
The following sentences were added:
"While MPP^+ also decreased the p13 protein levels as well as *p13* mRNA levels, rotenone treatment did not decrease p13 protein levels (Fig EV4C and D). The reason for the discrepancy is currently unclear. One possibility is that rotenone may inhibit p13 protein degradation through impairment of lysosomal functions [35]."

[Reviewer's comment]

4) Fig. 3D: Curiously, in the presence of CCCP treatment, the expression of p13 is dramatically reduced. Interestingly, p13 expression silencing appears to reduce the expression of PINK1 in the presence of CCCP. The authors did not provide possible explanation to these findings. Is there potential interaction between p13 and PINK1?

[Our Response]

We thank the reviewer for raising a very important issue. As suggested by the comment, we performed co-immunoprecipitation experiments for FLAG-tagged p13 and PINK1 in SH-SY5Y cells. We could not detect the physical interaction between p13 and PINK1 (data not shown). Instead, we found that the CCCP-induced reduction of ΔΨm was slightly reversed in p13 knockdown cells (Fig EV2F in the revised manuscript), which is likely to cause the slight decrease of PINK1 accumulation as compared to that in the control knockdown cells (Fig 3E in the revised manuscript), because the accumulation of PINK1 on the mitochondria is induced by the decreased levels of ΔΨm (Matsuda N et al., *J. Cell Biol.*, 2010. PMID: 20404107; Narendra DP et al., *Plos Biol.*, 2010. PMID: 20126261). These results suggest that the CCCP-induced PINK1 accumulation may not be directly linked to the p13 degradation. Regarding CCCP-induced reduction of p13 expression, we found that the CCCP treatment did not decrease *p13* mRNA levels (Fig EV2G in the revised manuscript) and that the CCCP-induced decrease in p13 levels was prevented by bafilomycin A1, an autophagy inhibitor (Fig EV2H in the revised manuscript), suggesting that p13 may be partially degraded by CCCP-mediated autophagy. Please also see the response to the comment #3-10. We have added the relevant descriptions to the Results and Discussion as follows:

The Results and Discussion (page 9, line 24 - page 10, line 2)
The following sentences were added:
"We also found that the CCCP-induced reduction of ΔΨm was slightly reversed in p13 knockdown cells (Fig EV2F), which is likely to cause the slight decrease of PINK1 accumulation as compared to that in the control knockdown cells (Fig 3E). In addition, we found that CCCP treatment did not decrease *p13* mRNA levels (Fig EV2G) and that the CCCP-induced decrease in p13 levels was prevented by bafilomycin A1, an autophagy inhibitor (Fig EV2H), suggesting that p13 may be

partially degraded by CCCP-mediated autophagy ahead of the other mitochondrial proteins such as Tim23 and Hsp60 (Fig EV2C and D)."

[Reviewer's comment]

5) It is also curious why rotenone despite reducing mitochondria membrane potential (Fig 2C) did not lead to an increase in PINK1 levels.

[Our Response]

We thank the reviewer for raising a very important issue. To answer the question, we compared the ΔΨm of both rotenone- and CCCP- treated cells and found that, while CCCP considerably reduced ΔΨm, rotenone mildly reduced ΔΨm (Fig EV2E in the revised manuscript). Since PINK1 accumulates on the mitochondria following the loss of ΔΨm [19], the rotenone-induced depolarization of ΔΨm may not be sufficient to trigger PINK1 accumulation. We have added relevant descriptions to the Results and Discussion as follows:

The Results and Discussion (page 9, lines 20-24)

The following sentences were added:

"The underlying mechanism of these results remains unclear, but we found that rotenone mildly reduced ΔΨm compared to CCCP (Fig EV2E). Since PINK1 accumulates on the mitochondrial outer membrane following the loss of ΔΨm [19], the rotenone-induced depolarization of ΔΨm may not be sufficient to trigger PINK1 accumulation."

[Reviewer's comment]

6) Fig. 3D: Full length PINK1 that accumulates in the presence of CCCP should be around 62-64 kD (not 50kD).

[Our Response]

We apologize for the inadvertent error. We carefully performed western blotting again and detected full-length PINK1, approximately 62 kDa (Fig 3E, EV2C and D in the revised manuscript).

[Reviewer's comment]

7) Fig. 3C: Show immunoblot for complex I. Same for Fig. 4D.

[Our Response]

As suggested by this comment, we performed western blotting for nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1β subcomplex 8 (NDUFB8), one of the mitochondrial complex I subunits, to confirm that the antibody we used actually precipitated the mitochondrial complex I. We found that the antibody, but not the control IgG, precipitated NDUFB8 as well as p13 (Fig 3B, 3C and 4F in the revised manuscript). We have amended the Results and Discussion as follows:

The Results and Discussion (page 8, lines 21-28)

"We also found that overexpressed p13 was co-localized and co-precipitated with complex I proteins in SH-SY5Y cells (Fig 3B and C)."

was changed to

"We next examined the co-immunoprecipitation study to show the direct interaction between p13 and mitochondrial complex I using an anti-complex I immunocapture antibody. We found that overexpressed p13 co-precipitated well with complex I, as revealed by an immunoblot for nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1β subcomplex 8 (NDUFB8), a mitochondrial complex I subunit, in SH-SY5Y cells (Fig 3B). Endogenous p13 also co-precipitated with complex I proteins in SH-SY5Y cells (Fig 3C). Immunocytochemistry showed that overexpressed p13 was co-localized with mitochondrial complex I (Fig EV2A)."

[Reviewer's comment]

8) Fig. 3E: Show p13 immunoblot.

[Our Response]

As suggested by the comment, we added the endogenous p13 immunoblot in Fig 3F of the revised manuscript.

[Reviewer's comment]

9) Where shRNA studies were performed, it is common to have at least two shRNA species to rule out off-target effects. Ideally, a genetic rescue experiment with a shRNA-resistant p13 cDNA should be included.

[Our Response]

We agree with the reviewer's comment. In response to the comment, we constructed another shRNA construct (p13 shRNA #2) whose target sequence is different from that of the p13 shRNA construct we have already used. We investigated the effect of p13 knockdown by shRNA #2 on depolarization of ΔΨm, apoptosis and complex I activity under toxin-induced conditions and found that the results were consistent with what was obtained using the original p13 shRNA construct (Fig EV1C–F in the revised manuscript). We have added the following sentences to the Results and Discussion.

The Results and Discussion (page 7, lines 20-22)

The following sentences were added:

"Similar results were obtained using another shRNA construct (p13 shRNA #2) targeting a different region of *p13* (Fig EV1C–E), excluding the possible off-target effect of shRNA."

[Reviewer's comment]

10) TH-positive neurons should be quantified via stereology, which provides an unbiased measurement and is a standard method for PD animal studies. Typically, Nissl staining is provided alongside. It would also be informative to show the striatal dopaminergic innervation.

[Our Response]

We appreciated this valuable suggestion. In response to the comment, we stereologically counted total numbers of TH-positive neurons in the substantia nigra with Stereo Investigator software (MBF Bioscience, Williston, VT, USA) using a fractionator (Fig 4B in the revised manuscript). We again observed that MPTP-induced reduction of the number of TH-positive neurons was almost completely reversed in *p13* $^{+/-}$ mice compared with *p13* $^{+/+}$ mice. In addition, as suggested, we also performed Nissl staining of the substantia nigra (Fig 4B in the revised manuscript). Furthermore, we measured the optical density of TH-positive fibres in the striatum and found that MPTP-induced reduction in the optical density of TH-positive fibres was not restored in *p13* $^{+/-}$ mice compared with *p13* $^{+/+}$ mice (Fig EV5F in the revised manuscript). As previously reported [37], these results suggest that restoration of dopaminergic function in the substantia nigra is important for the mitigation of MPTP-induced motor dysfunction. We have accordingly amended the relevant descriptions in the Results and Discussion as follows:

The Results and Discussion (page 11, lines 15-25)

"We then examined the MPTP-induced degeneration of dopaminergic neurons in the substantia nigra of *p13*+/+ and *p13*+/- mice and observed that MPTP-induced reduction in the number of tyrosine hydroxylase-expressing (TH+) cells was almost completely reversed in *p13*+/- mice compared with *p13*+/+ mice (Fig 4C)."

was changed to

"We then examined the MPTP-induced degeneration of dopaminergic neurons in the substantia nigra of *p13*$^{+/+}$ and *p13*$^{+/-}$ mice by using Stereo Investigator software with a fractionator (MBF Bioscience). We found that the MPTP-induced reduction in the number of tyrosine hydroxylase-expressing (TH^{+}) cells was almost completely reversed in *p13*$^{+/-}$ mice compared with *p13*$^{+/+}$ mice (Fig 4B). In vehicle-treated mice, there was no difference in the number of TH^{+} cells between *p13*$^{+/+}$ and *p13*$^{+/-}$ mice (Fig 4B). Furthermore, we measured the optical density of TH^{+} fibres in the striatum and found that MPTP-induced reduction in the optical density of TH^{+} fibres was not reversed in *p13*$^{+/-}$ mice compared with *p13*$^{+/+}$ mice (Fig EV5F). As previously reported [37], these results suggest that restoration of dopaminergic function in the substantia nigra is important for the improvement of MPTP-induced motor dysfunction."

[Reviewer's comment]

11) Fig. 4 & EV3: What is the level of p13 in various regions of the mouse brain following MPTP treatment?

[Our Response]

In response to the comment, we investigated the *p13* mRNA levels in various regions of the mouse brain following MPTP treatment. We found that MPTP significantly decreased *p13* mRNA levels selectively in the midbrain among examined samples (Fig EV3D in the revised manuscript). We also performed western blot analysis and found that p13 expression was decreased in the midbrain of MPTP-treated mice compared to that of vehicle-treated mice (Fig EV3E in the revised manuscript). These data are consistent with the vulnerability of midbrain dopaminergic neurons to MPTP [15]. We have amended the relevant descriptions in the Results and Discussion as follows:

The Results and Discussion (page 10, lines 13-19)
"Using the MPTP-induced PD model, we next examined whether MPTP application changes *p13* mRNA expression levels in the striatum and midbrain, including the substantia nigra, both of which are critically damaged in PD. We found that MPTP significantly decreased *p13* levels selectively in the midbrain among examined samples (Fig 4A)."
was changed to
"Using the MPTP-induced PD model, we next examined whether MPTP application changes *p13* expression levels in the various regions of the brain. We found that MPTP significantly decreased *p13* mRNA expression levels selectively in the midbrain among the examined samples (Fig EV3D). We also performed western blot analysis and found that p13 expression in the midbrain of MPTP-treated mice was decreased compared to that of vehicle-treated mice (Fig EV3E). These data are consistent with the vulnerability of midbrain dopaminergic neurons to MPTP [15]."

[Reviewer's comment]

12) The authors suggested that "p13 knockdown could be a candidate drug target for PD" (p. 10). It appears that targeting p13 might be challenging as the authors suggest that this needs to be done before disease progression. Moreover, in their previous report, the authors showed that p13 overexpression is beneficial against type 2 diabetes (Higashi et al., 2015 BBRC). How the authors propose to implement this strategy in the clinical setting for PD is therefore unclear.

[Our Response]

We agree with the reviewer's concern that targeting p13 might be challenging in the clinical setting for PD. In particular, we currently have no experimental data suggesting whether p13 knockdown after disease progression is effective for PD; further studies are needed to propose a new therapeutics targeting p13 for PD. Therefore, we have weakened our claims that p13 knockdown could be a candidate drug target for PD. We have accordingly amended the relevant descriptions in the Results and Discussion as follows:

The Results and Discussion (page 14, lines 3-6)
"In conclusion, our findings should help to explain the molecular pathogenesis of PD and to develop mitochondria-based drugs for PD. In particular, in view of the effects of p13 knockdown in the *in vitro* and *in vivo* PD models used in this study, p13 knockdown could be a candidate drug target for PD. We believe that p13 is an ideal drug target because p13 knockdown seems to affect mitochondrial function specifically under disease conditions but not under basal conditions."
was changed to
"In conclusion, we demonstrated that targeted knockdown of the new mitochondrial protein p13 prevents mitochondrial dysfunction and dopaminergic neuronal death in both *in vitro* and *in vivo* PD models. Our findings should help to explain the molecular pathogenesis of PD. Further studies are needed to propose a new PD therapeutic that targets p13."

Responses to Reviewer #3:

In this manuscript, Inoue et al examine the mitochondrial function of p13 (a protein that they recently identified) in SHSY5Y neuroblastoma cells. Using a p13-deficient mice, they further show that the heterozygous animals are resistant against MPTP-induced motor deficits and dopaminergic neuronal loss.
The manuscript is well-written but a lot of information is missing (from the legends in particular) making it tedious for the reader. The work in mice (in particular the p13 KO mice) is interesting and could be of interest for the EMBO readership. Unfortunately however, most of the experiments have been performed in SHSY5Y neuroblastoma cells.

[Our Response]

We appreciate the reviewer's positive evaluation of our work and thank her/him for the constructive suggestions. Our point-by-point responses to the comments are as follows. Furthermore, as suggested by the reviewer, we thoroughly rewrite the manuscript (especially the Figure Legends) so that the reader can easily understand the results.

[Reviewer's comment]

1) - In Figure 1A, I assume that the authors have used a FLAG antibody to detect p13 (information missing from the legends)? In Figure 2 and in Figure 4, the authors use an antibody against p13. Can they use it in immunofluorescence to confirm mitochondrial localisation of endogenous p13?

[Our Response]

We apologize for the omission of this information from the legends. In the immunofluorescent experiments (Fig 1A), we used anti-p13 antibody to detect the localization of p13. In the mock virus-infected cells, we could not detect endogenous p13 in the immunofluorescent experiments using the anti-p13 antibody, although the antibody can detect endogenous p13 in western blotting. Instead of the immunofluorescent experiments, subcellular fractionation was carried out to resolve the cellular localization of endogenous p13. We found that endogenous p13 localizes in the mitochondria, especially in the mitochondrial matrix (Fig 2A, B and EV3B in the revised manuscript) (Please see our response to reviewer's comment #2-1). We have amended the relevant descriptions in the Figure legends and Results and Discussion as follows:

Figure legends (page 28, lines 5-7)

"The co-localization of overexpressed p13 and Hsp60, a mitochondrial matrix protein, in SH-SY5Y cells infected with lentiviral vectors expressing mock or FLAG-tagged p13 (p13 o/e)."

was changed to

"Co-localization of overexpressed p13 and Hsp60, a mitochondrial matrix protein, in p13-infected SH-SY5Y cells. Overexpressed p13 was detected using an antibody against p13."

The Results and Discussion (page 7, lines 2-11)

The following sentences were added:

"To resolve the cellular localization of endogenous p13, we first performed subcellular fractionation experiments and observed that endogenous p13 was most abundant in the mitochondria-enriched fraction (Fig 2A and EV3B). Furthermore, to characterize the intramitochondrial localization of endogenous p13, we used digitonin fractionation, in which mitochondria were treated with various concentrations of digitonin for progressive membrane solubilization. We found that p13 showed a similar resistance to digitonin compared with the mitochondrial matrix marker Hsp60 (Fig 2B). Tom20 and Tim23, which are mitochondrial outer and inner membrane markers, respectively, are more sensitive to higher concentrations of digitonin than p13 or Hsp60 is (Fig 2B). These results suggest that p13 is mainly localized in the mitochondrial matrix."

[Reviewer's comment]

2) - In Figure 1D, the PARP cleavage blot is not terribly convincing (the western blot is not representative of the histogram). The authors should confirm that apoptosis is increased in their model using other methods.

[Our Response]

We thank the reviewer for raising a very important issue. In response to the comment, we examined the number of apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick-end labelling (TUNEL) method. In accordance with the PARP cleavage, we found that overexpression of p13 increased TUNEL-positive cells under basal condition and exacerbated the rotenone-induced increase in TUNEL-positive cells (Fig 1E in the revised manuscript). Furthermore, p13 knockdown significantly prevented the rotenone-induced increase in TUNEL-positive cells (Fig 2E in the revised manuscript). As expected by the results of the PARP cleavage assay (Fig 2B in the revised manuscript), p13 knockdown did not affect the percentage of TUNEL-positive cells under the basal condition (Fig 2E in the revised manuscript). We have accordingly amended the Results and Discussion section as follows. (Please see our response to the reviewer's comment #2-1.)

The Results and Discussion (page 6, lines 21-24)
The following sentences were added:
"We also applied the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick-end labelling (TUNEL) method to detect apoptotic cells. We found that overexpression of p13 increased the number of TUNEL-positive cells under basal conditions and exacerbated the rotenone-induced increase in TUNEL-positive cells (Fig 1E)."

The Results and Discussion (page 7, lines 16-20)
"In contrast to control knockdown by scrambled shRNA transfections, p13 knockdown significantly prevented the rotenone-induced decrease in ΔΨm and increase in cleaved PARP (Fig 2C and D). In the basal condition, the fluorescence intensity of TMRE and the cleavage of PARP were unchanged by the p13 knockdown (Fig 2C and D)."
was changed to
"In contrast to control knockdown, p13 knockdown significantly prevented the rotenone-induced decrease in ΔΨm and increases in cleaved PARP and TUNEL-positive cells (Fig 2C–E). In the basal condition, the p13 knockdown did not affect the fluorescence intensity of TMRE, the cleavage of PARP or the percentage of TUNEL-positive cells (Fig 2C–E)."

[Reviewer's comment]
3) In Figure 1D, did the authors use a FLAG or a p13 antibody (again, this isn't clear from the legend)? What is the band in the mock lane? Why is it not present in the p13 o/e lane?

[Our Response]
We again apologize for omitting important information from the legends. Since we used anti-p13 antibody in western blotting to determine the level of p13 expression (Fig 1D in the revised manuscript), we assume that the band in the mock lane was endogenous p13. Owing to the FLAG tag, the mobility of overexpressed FLAG-tagged p13 was decreased compared with that of endogenous p13. Currently, it is unclear why the endogenous p13 was decreased in the p13 overexpressed cells. One possibility is that overexpressed p13 might occupy the mitochondrial compartment, resulting in perturbation of the mitochondrial localization of endogenous p13 and degradation of endogenous p13. In addition to the figure legend, the representative blot in Fig 1D in the original manuscript seems to be confusing. Therefore, we have changed the representative image (Fig 1D in the revised manuscript) and amended the relevant descriptions in the Figure legends as follows:

Figure legends (page 28, lines 12-17)
"Expression of cleaved PARP, p13 and β-actin analysed by western blotting 96 h after infection of lentiviral vectors expressing mock or FLAG-tagged p13 (p13 o/e). Representative images are shown on the *left*. The band intensities of cleaved PARP were normalized to those of β-actin (*right*)."
was changed to
"Exacerbated rotenone-induced increase in PARP cleavage in p13-infected SH-SY5Y cells. Levels of cleaved PARP, p13 and β-actin were analysed by western blotting. Representative images (left) and the quantification of the band intensities of cleaved PARP (right). Total cell lysates were subjected to western blotting with antibodies against PARP, p13 and β-actin. p13 was detected using an antibody against p13. The levels of cleaved PARP were normalized to those of β-actin."

[Reviewer's comment]
4) What is the effect of a rotenone treatment on TMRE, Mito Tracker Green and PARP cleavage in SHSY5Y cells over-expressing p13?

[Our Response]
We thank the reviewer for raising a very important issue. In response to the comment, we investigated the effect of a rotenone treatment on TMRE, MitoTracker Green, PARP cleavage and TUNEL staining in p13-overexpressing SH-SY5Y cells. We found that p13 overexpression exacerbated the rotenone-induced decrease in fluorescent intensity of TMRE, but not of MitoTracker Green (Fig 1B and C in the revised manuscript). We also found that p13 overexpression significantly worsened rotenone-induced apoptosis as revealed by PARP cleavage and TUNEL staining (Fig 1D and E in the revised manuscript). These results support our notion that p13 overexpression induces mitochondrial dysfunction and apoptosis. Accordingly, we have amended the relevant descriptions in the Results and Discussion as follows:

The subheading in the Results and Discussion (page 6, lines 3-4)
"p13 overexpression induces mitochondrial dysfunction and apoptosis in SH-SY5Y cells"
was changed to
"p13 overexpression exacerbates rotenone-induced mitochondrial dysfunction and apoptosis in SH-SY5Y cells."

The Results and Discussion (page 6, lines 12-14)
The following sentence was added:
"The ΔΨm decrease induced by rotenone, a mitochondrial complex I inhibitor, was exacerbated in p13-overexpressed SH-SY5Y cells (Fig 1B)."

The Results and Discussion (page 6, lines 14-17)
"The signal of MitoTracker Green FM, which localizes to mitochondria regardless of ΔΨm, did not differ between mock- and p13-transfected cells (Fig 1C), suggesting that p13 overexpression does not affect mitochondrial mass."
was changed to
"The signal of MitoTracker Green FM, which localizes to mitochondria regardless of ΔΨm, did not differ between mock- and p13-overexpressed cells under basal or rotenone-treated conditions (Fig 1C), suggesting that p13 overexpression does not affect mitochondrial mass."

The Results and Discussion (page 6, lines 19-21)
"We observed that p13 overexpression significantly increased the levels of PARP cleavage (Fig 1D)."
was changed to
"We observed that p13 overexpression significantly increased the levels of PARP cleavage in both the vehicle- and the rotenone-treated cells (Fig 1D)."

The Results and Discussion (page 6, lines 21-24)
The following sentences were added:
"We also applied the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-uridine triphosphate (dUTP) nick-end labelling (TUNEL) method to detect apoptotic cells. We found that overexpression of p13 increased the number of TUNEL-positive cells under basal conditions and exacerbated the rotenone-induced increase in TUNEL-positive cells (Fig 1E)."

[Reviewer's comment]

5) The authors state, rightly so, that "In the basal condition, the fluorescence intensity of TMRE and the cleavage of PARP were unchanged by the p13 knockdown (Fig 2C and D)". How do they reconcile that with the fact that p13 o/e affects TMRE and PARP cleavage in basal conditions (Figure 1).

[Our Response]

We thank the reviewer for raising a very important issue. Currently it is unclear why, in contrast to p13 overexpression, p13 knockdown did not affect the fluorescence intensity of TMRE and the cleavage of PARP in the basal condition. We assume, however, several possibilities to reconcile the discrepancy. 1) A small amount of residual p13 protein after p13 knockdown may keep the mitochondrial function in the basal condition. 2) Other mitochondrial factors may compensate for the effect of p13 knockdown in the basal condition. These possibilities are supported by the previous studies showing that overexpression of a mitochondrial factor, hFis1 induces apoptosis, although hFis1 knockdown does not affect apoptosis (Yu T et al., *J. Cell Sci.*, 2005. PMID: 16118244; Lee YJ et al., *Mol. Biol. Cell*, 2004. PMID: 15356267). The relevant descriptions have been added as follows:

The Results and Discussion (page 8, lines 6-11)
The following sentences were added:
"Currently it is unclear why, in contrast to p13 overexpression, p13 knockdown did not affect the fluorescence intensity of TMRE and the cleavage of PARP in the basal condition. We assume, however, several possibilities to reconcile the discrepancy. 1) A small amount of residual p13 protein after p13 knockdown may keep mitochondrial function in the basal condition. 2) Other mitochondrial factors may compensate for the effect of p13 knockdown in the basal condition."

[Reviewer's comment]

6) In Figure 2E, the rescue is very modest. The authors forgot to add the stats between Scr shRNA and p13shRNA + p13 o/e. If there is still a significant difference (which the histogram suggests), claiming that "the restoration of p13 expression significantly reversed the p13 knockdown-induced protective effects on ΔΨm and apoptosis in rotenone-treated cells (Fig 2E and F)" is an overstatement.

[Our Response]

We appreciated the reviewer's comment. After quantification of the fluorescent intensity of TMRE between scr shRNA and p13 shRNA + p13 o/e, there is still a significant difference between scr shRNA and p13 shRNA + p13 o/e, as noted by the reviewer (Fig 2F in the revised manuscript). Therefore, we have weakened our claim that p13 overexpression significantly reversed the p13 knockdown-induced protective effects on ΔΨm in rotenone-treated cells.

The Results and Discussion (page 7, line 29 - page 8, line 4)

"Importantly, in rescue experiments, the restoration of p13 expression reversed the p13 knockdown-induced protective effects on ΔΨm and apoptosis in rotenone-treated cells (Fig 2E and F)."

was changed to

"Importantly, in rescue experiments (Fig 2F and G), the restoration of p13 expression in p13 knockdown cells significantly reversed the protective effects of p13 knockdown on PARP cleavage by rotenone (Fig 2G), but the effect of the restoration of p13 expression on the rotenone-induced decrease in ΔΨm was modest (Fig 2F)."

[Reviewer's comment]

7) In Figure 2F, again the PARP cleavage is very modest on the Western blot (not representative of the histogram)

[Our Response]

In response to this comment, we replaced the image of the PARP cleavage with a new image representing the histogram (Fig 2G in the revised manuscript).

[Reviewer's comment]

8) Figure 3B is not very informative and could be moved to supplementary data.

[Our Response]

In response to this comment, Fig 3B in the original manuscript was moved to Fig EV2A in the revised manuscript.

[Reviewer's comment]

9) In figure 3C, all the loading controls are missing. The authors should provide the blot of the input, and the supernatant before/after IP. The authors should try to repeat the experiment with endogenous p13.

[Our Response]

In response to this comment, we provided the blots of the supernatant before and after IP (Fig 3B in the revised manuscript). In particular, we performed western blotting for nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1β subcomplex 8 (NDUFB8), one of the complex I subunits, to confirm that the antibody we used actually precipitated the mitochondrial complex I. Furthermore, we performed co-immunoprecipitation experiments with endogenous p13 and found that p13 interacted with mitochondrial complex I as revealed by immunoblotting for NDUFB8 (Fig 3C in the revised manuscript). The relevant descriptions were added as follows. Please see our response to the reviewer's comment #2-7.

The Results and Discussion (page 8, lines 21-28)

"We also found that overexpressed p13 was co-localized and co-precipitated with complex I proteins in SH-SY5Y cells (Fig 3B and C)."

was changed to

"We next examined the co-immunoprecipitation study to show the direct interaction between p13 and mitochondrial complex I using an anti-complex I immunocapture antibody. We found that

overexpressed p13 co-precipitated well with complex I, as revealed by an immunoblot for nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1β subcomplex 8 (NDUFB8), a mitochondrial complex I subunit, in SH-SY5Y cells (Fig 3B). Endogenous p13 also co-precipitated with complex I proteins in SH-SY5Y cells (Fig 3C). Immunocytochemistry showed that overexpressed p13 was co-localized with mitochondrial complex I (Fig EV2A)."

[Reviewer's comment]

10) Figure 3D and E: I assume the authors have used whole cell lysates (since they detect actin in all the samples)? I would suggest repeating the experiments with mitochondrial enriched fractions, as it seems that PINK1 accumulation is decreased in CCCP-treated p13 shRNA cells as compared to scr. How long were the cells treated with rotenone and CCCP for? The authors should do short treatment (to detect PINK1 accumulation), and longer treatments, to detect ubiquitination of outer membrane proteins (E.g Mfn) and disappearance of mitochondrial markers. How do the authors explain that p13 signal disappears in scr cells after CCCP treatment?

[Our Response]

We thank the reviewer for raising a very important issue. As suggested by the comment, we repeated the experiments with mitochondrial enriched fractions from the cells treated with CCCP for 24 or 48h and obtained essentially the same results as those using whole cell lysates (Fig EV2C, EV2D and 3E in the revised manuscript). We found that the 24 h treatment of cells with CCCP did not decrease *p13* mRNA levels (Fig EV2G in the revised manuscript) and that the CCCP-induced decrease in p13 levels was prevented by bafilomycin A1, an autophagy inhibitor (Fig EV2H in the revised manuscript), suggesting that p13 may be partially degraded by CCCP-mediated autophagy. Currently, it is unclear why p13 is degraded ahead of the other mitochondrial proteins such as Tim23 and Hsp60 (Fig EV2C and D in the revised manuscript), which will be addressed in future studies. We sincerely hope that the reviewer will agree with us on this point. Also, please see the response to the comment #2-4. We have added the relevant descriptions to the Results and Discussion as follows:

The Results and Discussion (page 9, line 24 - page 10, line 2)

The following sentences were added:

"We also found that the CCCP-induced reduction of ΔΨm was slightly reversed in p13 knockdown cells (Fig EV2F), which is likely to cause the slight decrease of PINK1 accumulation as compared to that in the control knockdown cells (Fig 3E). In addition, we found that the CCCP treatment did not decrease *p13* mRNA levels (Fig EV2G) and that the CCCP-induced decrease in p13 levels was prevented by bafilomycin A1, an autophagy inhibitor (Fig EV2H), suggesting that p13 may be partially degraded by CCCP-mediated autophagy ahead of the other mitochondrial proteins such as Tim23 and Hsp60 (Fig EV2C and D)."

[Reviewer's comment]

11) The work in the KO mice is interesting. Have the authors assessed TMRM, PARP cleavage, mitophagy etc in mouse neurons from the midbrain, and other brain regions. Experiments from Figures 1, 2 and 3 should be repeated in mice neurons.

[Our Response]

We thank the reviewer for raising a very important issue. We measured mitochondrial membrane potential (ΔΨm), apoptosis and autophagy by TMRE, TUNEL-staining and measuring the conversion of LC3-I to LC3-II, respectively using midbrain samples from $p13^{+/+}$ and $p13^{+/-}$ mice. Together with the data (Fig 4F-H in the revised manuscript), we obtained essentially the same results as were obtained with SH-SY5Y cells (Fig 4C-E in the revised manuscript). The relevant descriptions were added as follows:

The Results and Discussion (page 11, line 25 - page 12, line 1)

The following sentence was added:

Furthermore, we found that the percentage of TUNEL-positive cells was decreased in the substantia nigra of MPTP-treated $p13^{+/-}$ mice (Fig 4C). We also observed that the conversion of LC3 was not induced in the mouse substantia nigra by MPTP regardless of the difference of p13 expression (Fig 4D). Interestingly, we found that the rotenone-induced decrease in ΔΨm was slightly attenuated in isolated mitochondria from the midbrain of $p13^{+/-}$ mice (Fig 4E).

The Results and Discussion (page 12, lines 5-6)
The following sentence was added:
"These results argue that targeted knockdown of p13 protects against experimental parkinsonism *in vivo* as well as *in vitro*."

[Reviewer's comment]
12) It would be interesting to determine if p13 mRNA levels are decreased in brains from patients with sporadic Parkinson's disease.

[Our Response]
We agree with this valuable suggestion. We could obtain only three pairs of postmortem brains from PD patients and age-matched control subjects for this study. We measured the expression levels of *p13* and *β-actin* mRNAs in the frontal cortex of these postmortem brains by real-time RT-PCR. We found that the relative *p13* expression levels in the frontal cortex of PD patients tended to be lower than those in control subjects. These data may support our findings that the expression level of p13 mRNA was reduced by parkinsonian toxicants. However, the present result is necessarily preliminary mainly due to the small sample size. Therefore, we have added these data as a supplementary figure (Fig EV4G in the revised manuscript). The relevant descriptions were added as follows:

The Results and Discussion (page 13, lines 2-9)
The following sentence was added:
"To evaluate the role of p13 in PD pathogenesis, we examined the expression level of *p13* mRNA in the postmortem brain tissue of three PD patients and three age-matched control subjects. *p13* mRNA expression levels in the frontal cortex of the patients with PD tended to be lower than those of the control subjects (Fig EV4G). However, the present result is necessarily preliminary, mainly owing to the small sample size. The additional therapeutic potential of p13 reduction, such as lack of tolerance or prevention of the disease progression, would be evaluated via further comparative studies using PD patient samples."

[Reviewer's comment]
13) In Figure 4D, again all the loading controls are missing. For example, are the complex I protein levels the same in the +/+ and -/- mice?

[Our Response]
According to this comment, we provided the blots of the supernatant before and after IP (Fig 4F in the revised manuscript). In particular, we performed western blotting for nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1β subcomplex 8 (NDUFB8), one of the complex I subunits, to confirm that the antibody we used actually precipitate the mitochondrial complex I. Furthermore, we examined the expression levels of NDUFB8 and nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) iron-sulfur protein 4 (NDUFS4), both of which are the complex I subunits, and found that the complex I protein level in the brain of $p13^{-/-}$ mice was virtually identical to that of $p13^{+/+}$ mice (Fig EV5G in the revised manuscript). The relevant descriptions were added as follows:

The Results and Discussion (page 12, lines 6-8)
The following sentence was added:
"In addition, the complex I protein level in the brain of $p13^{-/-}$ mice was virtually identical to that of $p13^{+/+}$ mice (Fig EV5G)."

2nd Editorial Decision 14 December 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed referee reports as well as referee cross-comments. As you will see, all referees support the publication of your manuscript now. They only have a few more suggestions that I would like you to address before we can proceed with the official acceptance of your study. The referees agreed in the cross-comments that the patient brain data either need to be strengthened to be significant or removed, and that the data on PINK1/mitophagy/autophagy should be moved to expanded view

figures.

The manuscript currently has 4 main and 5 EV figures. You can have one more main figure in a scientific report, or you could have 6 EV figures, exceptionally.

Please move all methods to the main manuscript file. The character count does not include the methods. The Appendix file can then be deleted.

Figure 1A does not explain what the blue color shows, please add an explanation.

Please remove the red text from the article file when you upload the final version.

I would like to suggest a few minor changes to the abstract that needs to be written in present tense:

Mitochondrial dysfunction in the nigrostriatal dopaminergic system is a critical hallmark of Parkinson's disease (PD). Mitochondrial toxins produce cellular and behavioural dysfunctions resembling those in PD patients. Causative gene products for familial PD play important roles in mitochondrial function. Therefore, targeting proteins that regulate mitochondrial integrity could provide convincing strategies for PD therapeutics. We have recently identified a novel 13-kDa protein (p13) that may be involved in mitochondrial oxidative phosphorylation. In the current study, we examine the mitochondrial function of p13 and its involvement in PD pathogenesis using mitochondrial toxin-induced PD models. We show that p13 overexpression induces mitochondrial dysfunction and apoptosis. P13 knockdown attenuates toxin-induced mitochondrial dysfunction and apoptosis in dopaminergic SH-SY5Y cells via the regulation of complex I. Importantly, we generate p13-deficient mice using the CRISPR/Cas9 system and observe that heterozygous p13 knockout prevents toxin-induced motor deficits and the loss of dopaminergic neurons in the substantia nigra. Taken together, our results suggest that manipulating p13 expression may be a promising avenue for therapeutic intervention in PD.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The authors have addressed my questions adequately. the work with postmortem brain samples is obviously interesting, but as it stands also rather preliminary. It may be better at this stage to remove those data (or add more patient samples).

Referee #2:

Overall, I am satisfied with the revisions that the authors have made in response to my comments/suggestions. I am happy that they have either included new experimental data or improved previous data sets in their revised manuscript. Their efforts are well noted and I have no further comments.

Referee #3:

The authors have raised most of my concerns.

However the possible interaction of p13 with PINK1 (as questionned by reviewer 2 also), and the possible role of p13 in mitophagy remains a little confusing (+ in figure 4D, the authors assess LC3 lipidation in neurons, as opposed to mitophagy as requested by the reviewer). And the LC3 blots don't add much to the paper. I would suggest moving all the PINK1/mitophagy/autophagy data in the supplementary figures.

2nd Revision - authors' response 15 December 2017

We have revised the manuscript for finalization of the manuscript as follows:

- The patient brain data were removed.
- PINK1/mitophagy/autophagy data were moved to expanded view figures (Figure EV3A, D and EV6G).
- An explanation was added for the blue color in Figure 1A (page 30, line 7).
- All methods were moved to the main manuscript file. The Appendix file was deleted.
- The abstract was changed in response to the editor's comment.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: H i t o s h i H a s h i m o t o
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44860V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were decided based on previous studies using same technique.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, our sample size is similar to those generally used in the field.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There was no exclusion of samples from analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis was chosen appropriately to the best of our knowledge.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used non-parametric tests.
Is there an estimate of variation within each group of data?	Yes. Variation is shown in each figure as SEM.
Is the variance similar between the groups that are being statistically compared?	Not applicable.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

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http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in this study are shown (together with their catalog number) in Materials and Methods section of the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	There is no contamination of cell lines. Lenti-X 293T cell line was purchased commercially from Clontech.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have provided these detailed information in Materials and Methods section of the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal studies were approved by the Animal Care and Use Committee of Osaka University.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study protocol was approved by The Human Use Review Committees of Graduate School of Medicine, Osaka University, and Toneyama National Hospital, for the Protection of Human Subjects.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Informed consent was obtained from all subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.